Modulation of cytokine expression and lymphocyte subsets during the periparturient period in dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*

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ABSTRACT

The objective of this study was to evaluate cytokine gene expression and populations of lymphocyte subsets in periparturient dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP).

Blood was collected from noninfected, subclinical, and clinical MAP-infected dairy cows for 3 wks pre- to 4 wk post-calving. Expression of IFN-γ, IL-4, and IL-10 declined at calving compared with prepartum values in both control and infected cows. PBMCs isolated from infected cows had higher secretion of IFN-γ, IL-10, and TGF-β in the postpartum period compared with control cows. Flow cytometric analysis revealed that subclinical cows expressed a greater percentage of both CD8\(^+\) and γδ T-cells compared with the clinical cows. The percentage of CD4\(^+\) T-cells increased in clinical cows as parturition approached. Clinical cows expressed lower percentages of CD4\(^+\)CD5\(^{bright}\) and CD8\(^+\)CD5\(^{bright}\) compared with control cows, but greater percentages of CD5\(^{dim}\) cells for all lymphocyte subsets.

These data suggest that parturition is a very dynamic time period for host immunity, with potential for altered immunity to hinder the ability of dairy cows to thwart infectious diseases.

INTRODUCTION

On-farm observations suggest that dairy cows infected with MAP may demonstrate increased signs of clinical disease during the weeks following parturition. Parturition has a major impact on the number of lymphocytes and monocytes in the peripheral blood of healthy cows and alterations in these percentages play a significant role in the ability of the animal to respond to infection. The transition from the subclinical to clinical stage of MAP infection is characterized by a shift from cell-mediated (Th1) immunity to an antibody-mediated (Th2) humoral response. To date, limited research is available characterizing detailed aspects of periparturient immunosuppression in the dairy cow. Further, it is not clear what impact this time period and its associated stressors may have on host immunity in cows with paratuberculosis. Therefore, the objectives of this study were 1) to characterize cytokine gene expression and secretion, and 2) to determine percentages of lymphocyte subsets in periparturient dairy cows naturally infected with MAP.

MATERIALS AND METHODS

Twenty-three Holstein dairy cows were grouped according to infection status. These three groups consisted of 5 non-infected healthy cows, 14 cows naturally infected with MAP, but asymptomatic, and 4 naturally infected cows with clinical Johne’s disease. Non-infected control cows were characterized by repeated negative fecal cultures performed quarterly over a 3-5 year period and were negative on any serological assays (ELISA, IFN-γ) performed during this period.

Blood was collected from the jugular vein in 2x acid-citrate-dextrose (1:10) at -21, -14, -7, +1, +7, +14, and +28 days relative to parturition. Peripheral blood mononuclear cells (PBMCs) were isolated and cells were cultured at 1.4 x 10^6/mL in 48-well flat-bottomed plates with either medium alone (non-stimulated, NS), with concanavalin A (ConA; 10 µg/mL) or with MAP whole cell sonicate (MPS; 10 µg/mL) added to designated wells. Plates were incubated for 24 h at 39°C in 5% CO\(_2\) in a humidified atmosphere. After 24 h plates were removed and centrifuged at 400 x g for 5 min. Supernatants were removed and stored at -
20°C prior to cytokine measurement. The secretion of IFN-γ, IL-10, and TGF-β by PBMCs in the cell culture supernatant was determined by ELISA assay.

For flow cytometric analysis, PBMCs were resuspended in complete medium and 50 µL of the cell suspension was added to wells containing 50 µL of primary monoclonal antibody to CD4+, CD8+, γδ T-cells, B cells, and CD5. Analysis was conducted by gating on mononuclear cells based on forward and side scatter characteristics.

RNA was extracted from NS and ConA-stimulated PBMCs using the standard protocol for Trizol reagent. Total RNA was converted to first strand cDNA. For RT-PCR analysis, SYBR Green PCR master mixture, template cDNA, and gene-specific primers for IFN-γ, IL-12p35, IL-4, IL-10, and TGF-β were combined in a 20 µL reaction mixture. The β-actin gene was used as the control for calculation of dCt. RT-PCR was analyzed by using the 2^-ddCt method. The mean +1 DRTC dCt value within treatment was used as the reference expression point.

RESULTS AND DISCUSSION
Due to sampling error, we were not able to evaluate the cytokine gene expression data for the clinical cows in this study. When comparing control and subclinically infected cows, subclinical MAP infection did not have an effect on the gene expression of IFN-γ or IL-12. However, ConA-stimulated PBMCs from subclinical cows (14.36 ng/ml ± 1.6) tended (P < 0.06) to secrete more IFN-γ compared with the control cows (8.30 ± 2.2). Previous work in our laboratory showed that subclinical JD cows had greater IFN-γ expression compared with clinical cows (Stabel, 2000). Parturition had a significant effect on IFN-γ expression with declines in IFN-γ expression by NS (P < 0.05) (Fig. 1A) and ConA-stimulated (P < 0.01) PBMCs from both infection groups as parturition approached. This is in agreement with previous studies reporting a decline in Th1 cytokines at parturition (Shafer-Weaver et al., 1999). Interleukin-12 expression was not affected by infection or parturition (data not shown).

In addition to understanding the effects of parturition and MAP on the Th1 cytokines, we also sought to determine the role of the classical Th2 cytokines. In the current study, there was no effect of parturition or MAP infection on gene expression of Th2 or Th3 cytokines. Despite minimal differences in TGF-β expression, NS (Fig. 2A) and MPS-stimulated PBMCs isolated from clinical cows secreted (P < 0.05) more TGF-β during the immediate postpartum period compared with subclinical and control cows. In NS, PBMCs, control cows had greater (P < 0.01) IL-10 expression during the postpartum period (Fig. 1B). MPS-stimulated PBMCs from infected cows tended (P < 0.09) to have secreted more IL-10 than did the control cows at calving and during the postpartum period (Fig. 2B). Others have reported upregulation of IL-4 during the postpartum period, but this effect was not observed in the present study (Fig. 1C) (Shafer-Weaver and Sordillo, 1997).
**Fig. 2.** TGF-β and IL-10 secretion by PBMCs isolated from control (♦), subclinical (■), and clinical (▲) periparturient dairy cows naturally infected with MAP. A) TGF-β NS PBMCs. B) MPS-stimulated PBMCs. Significant differences between infection groups on a given day relative to calving are represented by asterisks ($P < 0.05$).

**Fig. 3.** Figure 3. Percentage of positive mononuclear cells from 24 h PBMCs isolated from control (♦), subclinical (■), and clinical (▲) periparturient dairy cows. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

The number of CD4$^+$ T-cells in the peripheral blood averaged 21.6% ± 2.1, 24.3% ± 1.3, and 25.7% ± 2.4 for control, subclinical, and clinical cows, respectively, with no differences noted between infection groups (Fig. 3A). The percentage of CD8$^+$ T-cells (Fig. 3B) ranged from 10-22% which is equivalent to published values for adult dairy cattle (Meglia et al., 2005). An interactive effect of infection group and parturition ($P < 0.01$) was observed with subclinically infected cows expressing 2-fold higher percentages of CD8$^+$ cells in the postpartum period. The percentage of γδ T-cells is greatest in the calf (40%) and gradually declines to approximately 5% of adult PBMCs (Hein and MacKay, 1991). In our study, clinical cows had much lower ($P < 0.01$) percentage of γδ T-cells compared with subclinical and control cows (Fig. 3C). An increase in this cell population was noted as parturition approached. In advanced stages of JD, antibody production by B-cells does little to protect the host from progressive MAP infection. Overall, neither infection status of the cows nor parturition had an effect on the overall percentages of B cells (data not shown).

CD5 is expressed on all bovine T cells and a subset of B cells. Both CD5 bright and dim populations were examined as a percentage of marker positive lymphocyte subsets. For CD4$^+$, CD8$^+$, and B cell subsets, clinical cows expressed the lowest percentage of CD5$^{bright}$ cells ($P < 0.01$) and the greatest percentage of CD5$^{dim}$ cells compared to the control ($P < 0.05$). B-cells expressing CD5 are capable of producing the Th2 cytokine, IL-10 (Gieni et al., 1997). The increased proliferation of CD5$^{dim}$ B cells by clinical Johne’s cows may directly inhibit the cell-mediated immune response by altering the cytokine microenvironment in favor of a Th2 response.

**CONCLUSION**
Results of this study indicate that parturition modulates IFN-γ and IL-10 expression in dairy cows. Furthermore, the percentages of both lymphocyte subsets and mononuclear cells are modulated by natural infection with MAP and by the periparturient period.

**REFERENCES**